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Lee et al.

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[54] **CULTURE AND PROCESS FOR PRODUCING ANTIBIOTIC LL-D42067ALPHA**

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Related U.S. Application Data

[62] Division of Ser. No. 593,160, Mar. 26, 1984, Pat. No. 4,551,533.

[51] Int. Cl.⁴ **C12P 17/16; C12N 1/20; C12R 1/03**

[52] U.S. Cl. **435/118; 435/253; 435/825**

[58] Field of Search **435/118, 253, 825**

[56] References Cited

FOREIGN PATENT DOCUMENTS

0105194 6/1982 Japan 435/825
0150390 9/1982 Japan 435/825

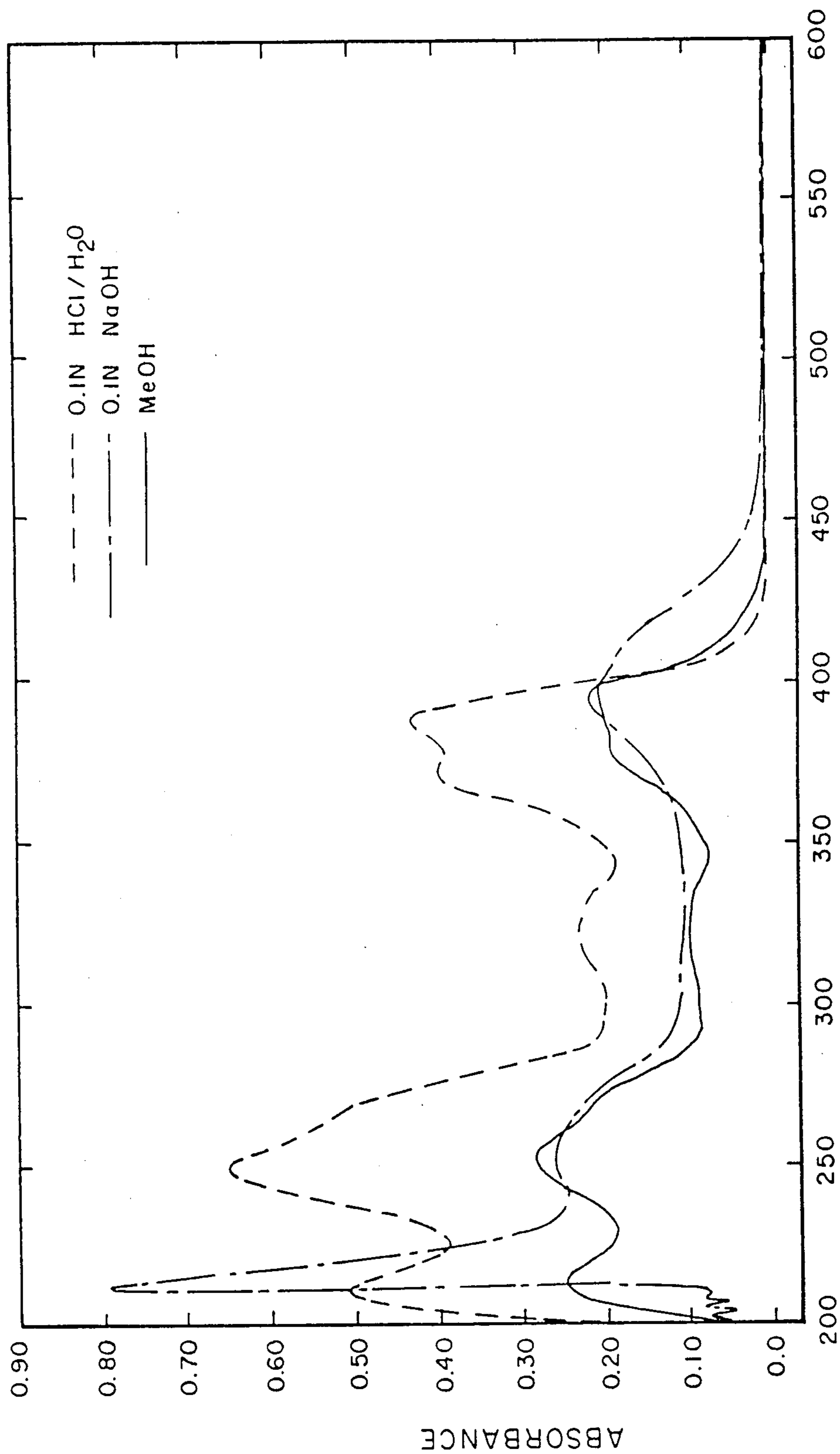
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[57] ABSTRACT

The invention is a process for the production by fermentation of the antibacterial and antiparasitic agents designated LL-D42067 α , to methods for its recovery and concentration from crude solutions and to processes for its purification. The invention includes within its scope the biologically pure culture of the antibiotic.

5 Claims, 5 Drawing Sheets

ULTRAVIOLET ABSORPTION SPECTRA OF LL-D42067 α
10ug/ml in MeOH, 0.1N HCl and 0.1N NaOH



WAVELENGTH (nm)

FIGURE I

INFRARED ABSORPTION SPECTRUM OF
LL-D42067 α (KBr disc)

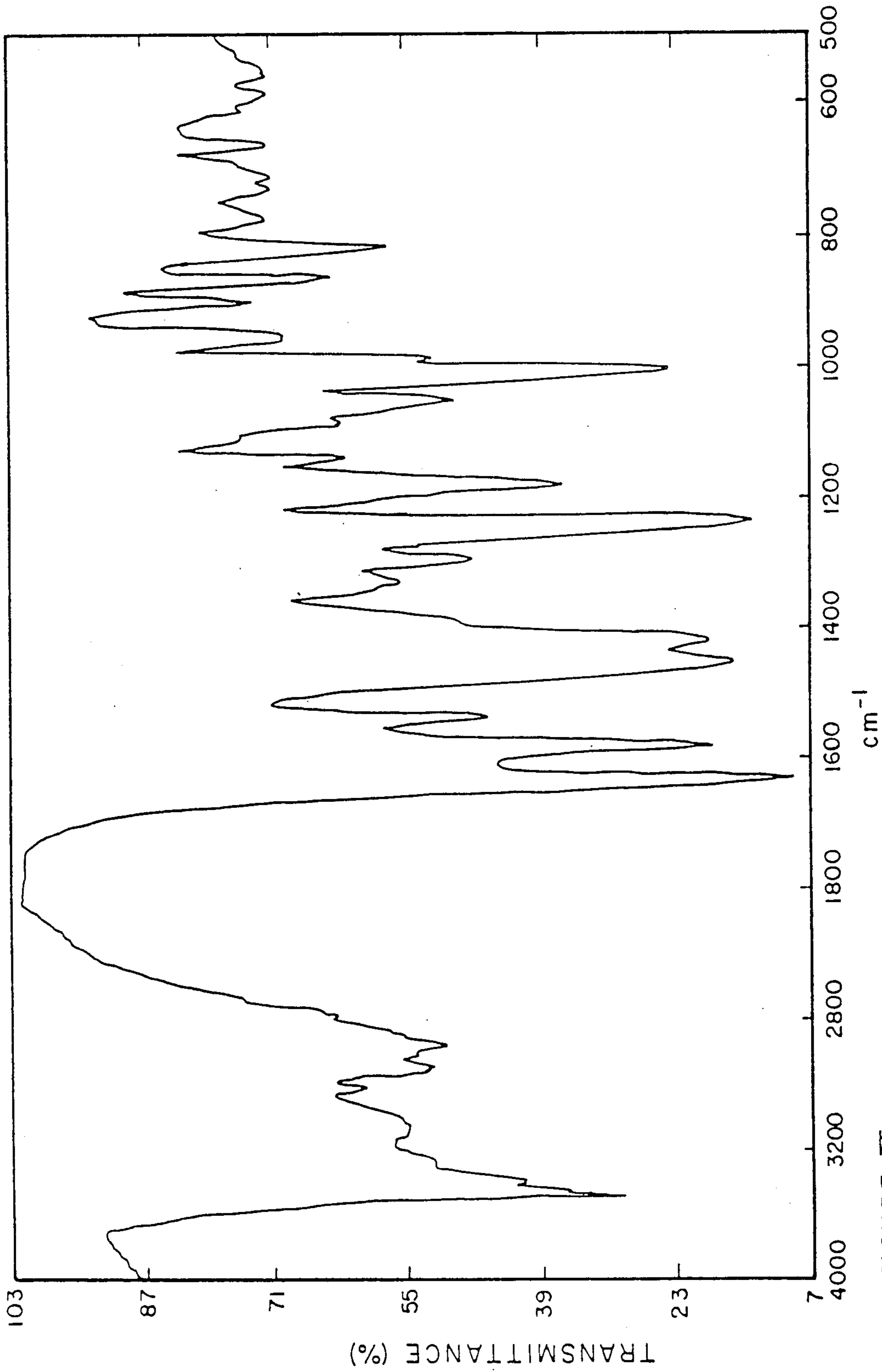


FIGURE II

PROTON NUCLEAR MAGNETIC RESONANCE SPECTRUM
OF LL-D42067 α IN CDCl₃ SOLUTION

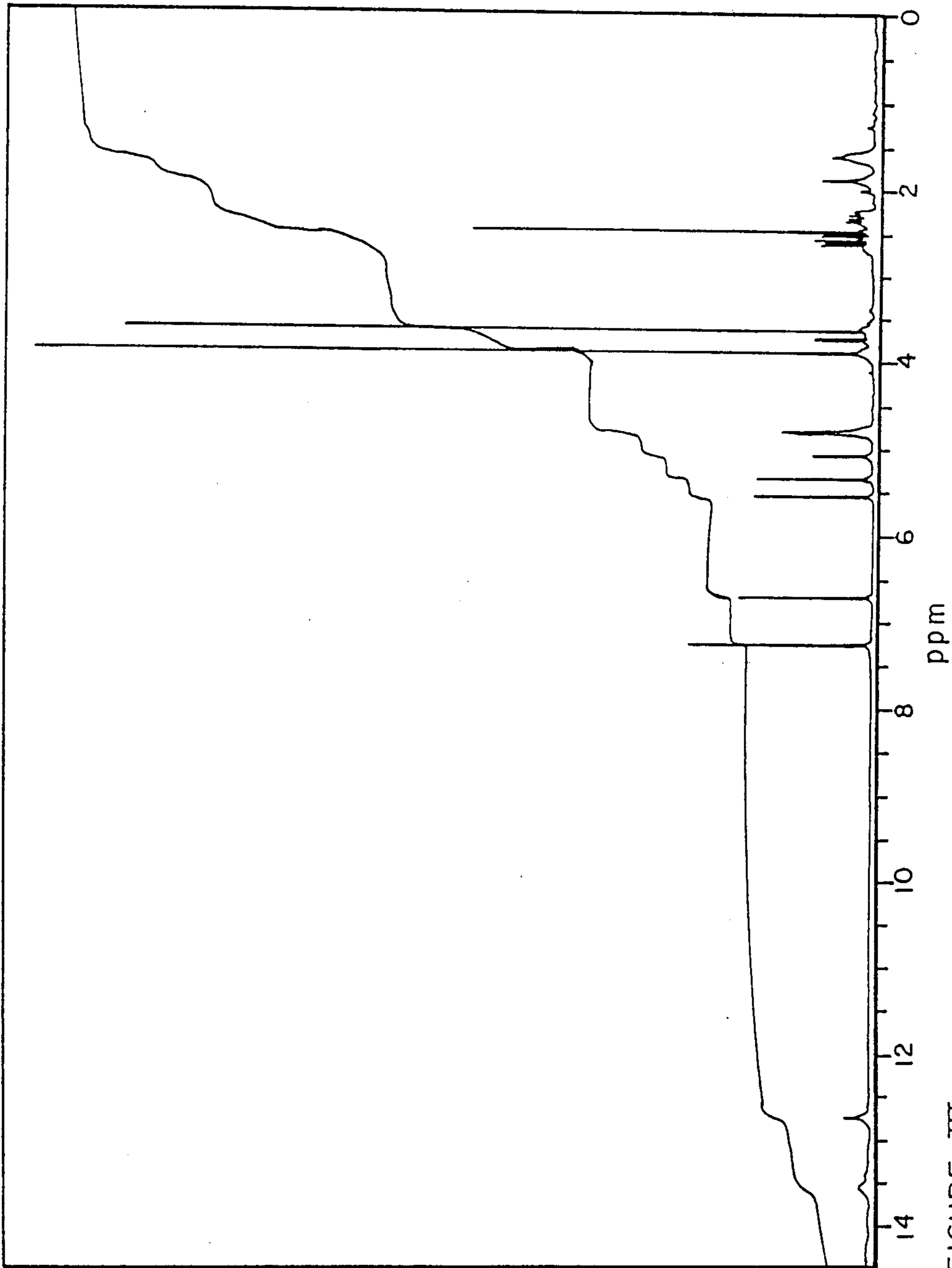


FIGURE III

CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTRUM
OF LL-D42067 α IN DMSO SOLUTION

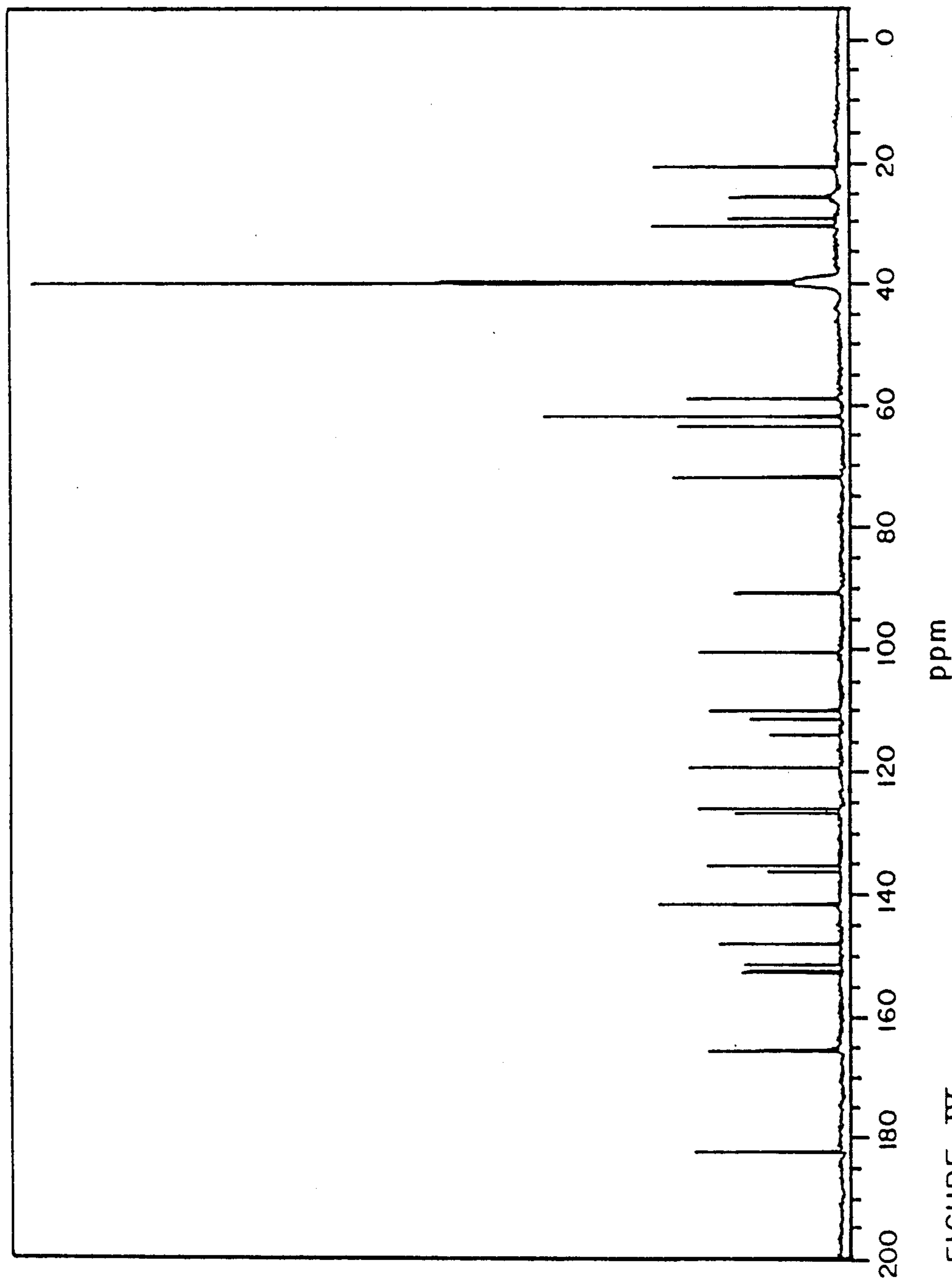


FIGURE IV

PROTON TO CARBON-13 CHEMICAL SHIFT CORRELATION
OF LL-D42067 α IN DMSO SOLUTION

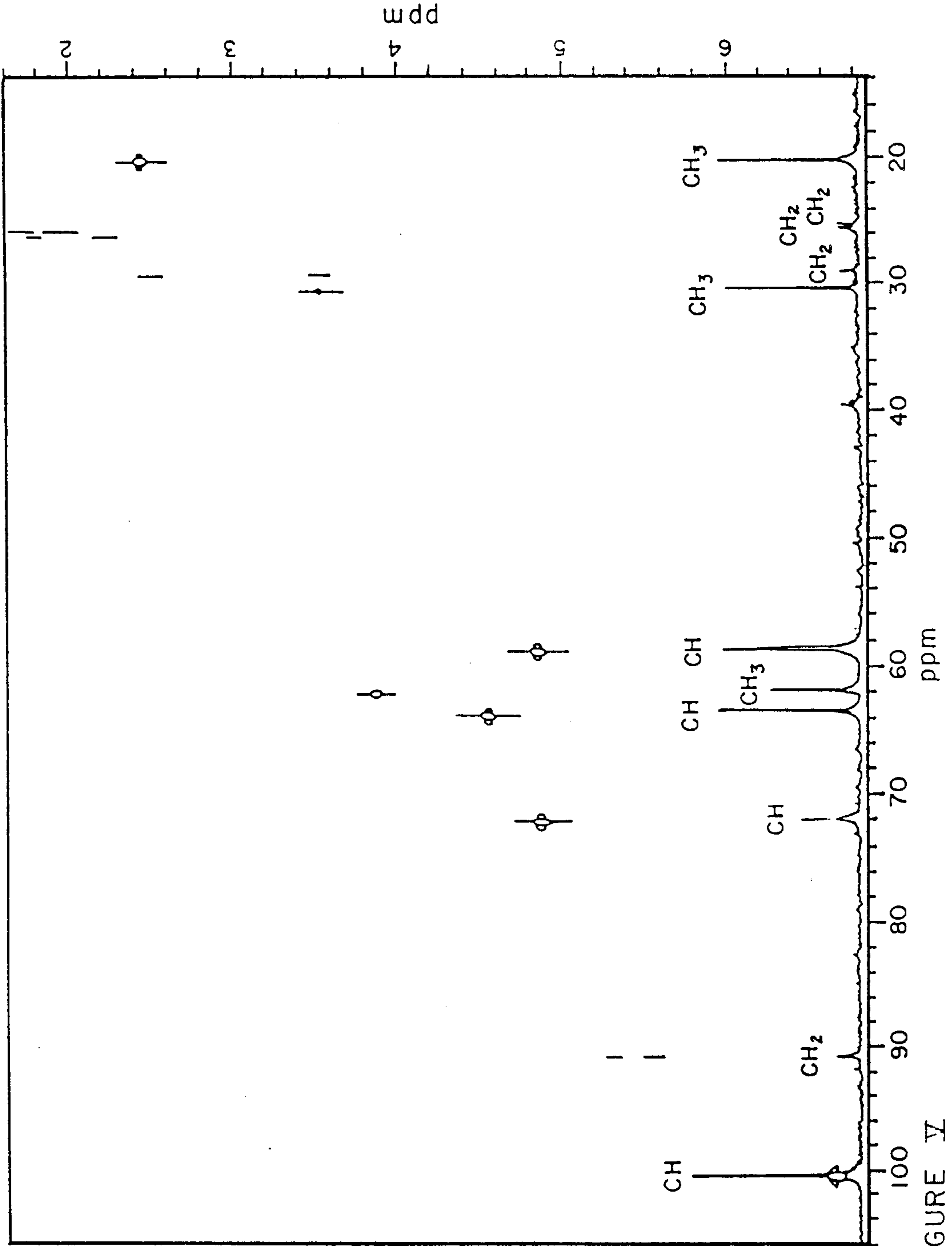


FIGURE V

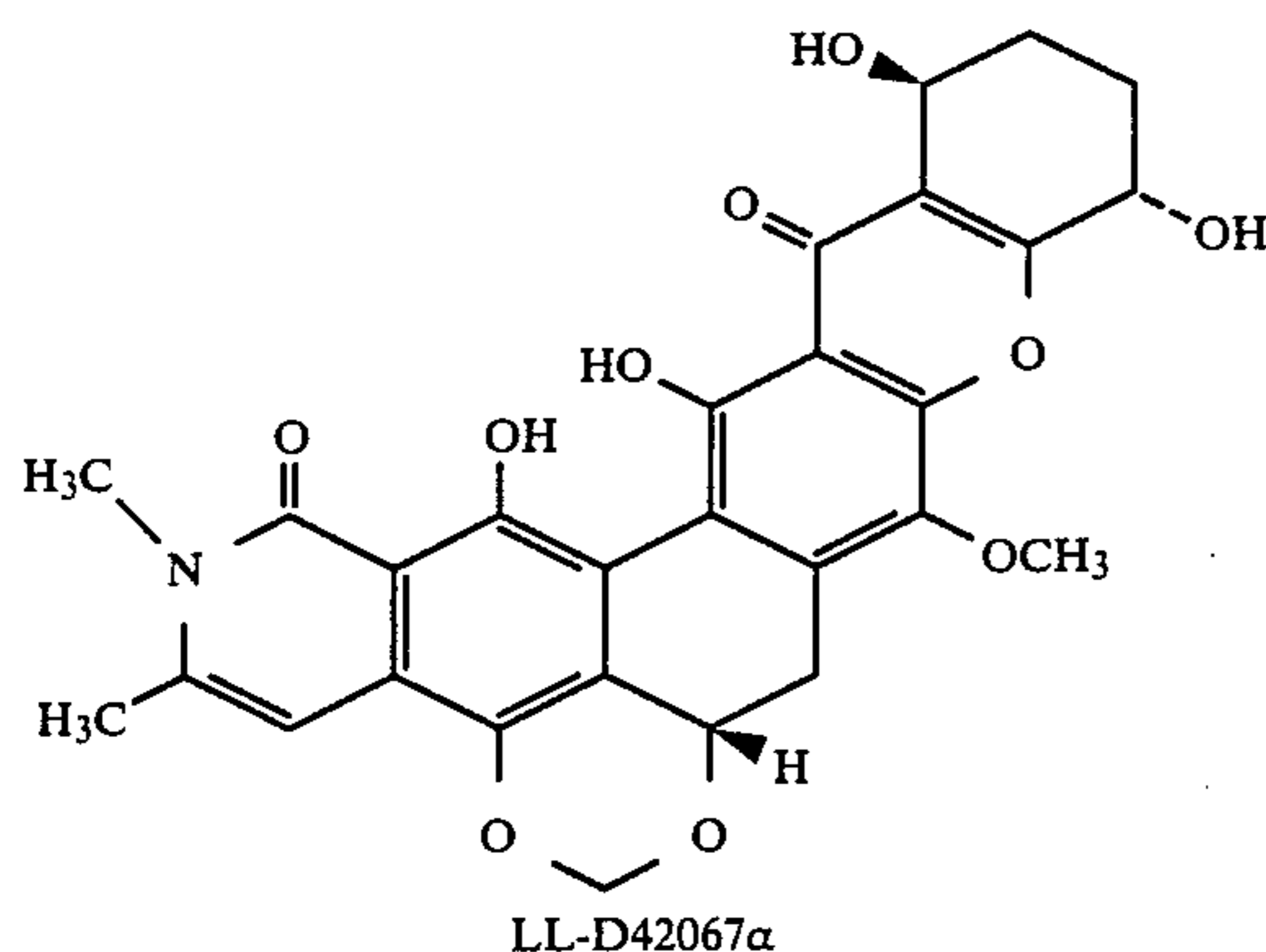
CULTURE AND PROCESS FOR PRODUCING ANTIBIOTIC LL-D42067ALPHA

This is a continuation of application Ser. No. 593,160, filed Mar. 26, 1984, which is now U.S. Pat. No. 4,551,533, issued Nov. 5, 1985.

SUMMARY OF THE INVENTION

This invention relates to a new antibacterial and anti-parasitic agent designated LL-D42067 α , to its production by fermentation, to methods for its recovery and concentration from crude solutions and to processes for its purification. The present invention includes within its scope the biologically pure culture of the antibiotic.

The structure and relative stereochemistry of LL-D42067 α have been elucidated by X-ray crystallography, and is shown below.



The physico-chemical characteristics of LL-D42067 α are described below:

- (1) Molecular weight: 535 (FAB-MS);
- (2) Molecular formula: C₂₈H₂₅NO₁₀;
- (3) Specific optical rotation: $[\alpha]_D^{26} = +836 \pm 40^\circ$ (C 0.3, DMF);
- (4) Ultraviolet absorption spectra: as shown in FIG. I

UV^{CH₃OH}_{MAX} = 215 nm (ϵ 13,200)
254 nm (ϵ 15,000)
320 nm (ϵ 5,100)
395 nm (ϵ 11,400)

UV^{0.1N HCl}_{MAX} = 213 nm (ϵ 27,100)
253 nm (ϵ 34,500)
321 nm (ϵ 12,200)
374 nm (ϵ 21,100)
389 nm (ϵ 22,900)

UV^{0.1N NaOH}_{MAX} = 217 nm (ϵ 42,100)
253 nm (ϵ 13,900)
312 nm (ϵ 5,700)
395 nm (ϵ 10,700);

(5) Infrared absorption spectrum: as shown in FIG. II (KBr disc): 1650, 1598, 1543, 1470, 1440, 1260, 1195, 1020 cm⁻¹;

(6) Proton nuclear magnetic resonance spectrum (CDCl₃): as shown in FIG. III, and described in Table I;

(7) Carbon-13 nuclear magnetic resonance spectrum (DMSO): as shown in FIG. IV and described in Table II; and

(8) Proton to carbon-13 chemical shift correlation map (DMSO): as shown in FIG. V.

TABLE I

Proton NMR Data for LL-D42067 α			
δ^*	No. of Hydrogen**	Multiplicity***	J (Hz)
1.88	2	m	
2.34	2	m	
2.45	3	s	
2.58	1	m	
3.62	3	s	
3.72	1	d,d	4.64, 14.22
3.88	3	s	
4.80	2	m	
5.08	1	m	
5.32	1	d	5.81
5.55	1	d	5.81
6.70	1	s	
12.76	1	s	
13.58	1	s	

*CDCl₃, ppm downfield from TMS.

**Spectrum in DMSO-d₆, shows two additional absorptions at 4.55(s) and 5.91 (d) ppm.

***s = singlet; d = doublet; t = triplet; m = multiplet.

TABLE II

Carbon-13 NMR Data for LL-D42067 α		
Carbon	Chemical Shift(ppm)*	Carbon Type
1	20.4	CH ₃
2	25.4	CH ₂
3	25.8	CH ₂
4	29.0	CH ₂
5	30.4	CH ₃
6	58.5	CH
7	61.6	CH ₃
8	63.3	CH
9	71.7	CH
10	90.4	CH ₂
11	100.0	CH
12	109.2	q**
13	109.7	q
14	111.0	q
15	113.7	q
16	119.0	q
17	125.8	q
18	126.6	q
19	134.9	q
20	135.3	q
21	136.1	q
22	141.3	q
23	147.9	q
24	151.1	q
25	152.5	q
26	165.4	q
27	165.6	q
28	182.3	q

*DMSO-d₆, ppm downfield from TMS.

**q = quarternary.

DETAILED DESCRIPTION OF THE INVENTION

The new antibacterial agent designated LL-D42067 α , is formed during the cultivation under controlled conditions of a new strain of a new subspecies of *Actinomadura madurae*. This new strain is maintained in the culture collection of the Medical Research Division, American Cyanamid Company, Pearl River, N.Y. as culture number LL-D42067. A viable culture of this new microorganism has been deposited with the Patent Culture Collection Laboratory, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. 61604, and has been added to its permanent

collection. It is freely available to the public in this depository under its accession number NRRL 15734.

Culture LL-D42067 was isolated from a soil sample from San Simao, Brazil. The culture was taxonomically characterized and was identified as a new subspecies of *Actinomadura madurae*, designated *Actinomadura madurae* subspecies *simaoensis*.

Observations were made of the cultural, physiological and morphological features of the culture in accordance with the methods detailed by Shirling and Gottlieb [Intern. J. System. Bacteriol., 16: 313-340 (1966)] and Gordon, et al. [Intern. J. System. Bacteriol., 24: 54-63 (1974)]. The chemical composition of the cell walls of the culture was determined using the method of Lechevalier, et al. [Adv. Appl. Microbiol., 14: 47-72 (1971)]. Details are recorded in Tables III-V, and a general description of the culture is given below. Underscored descriptive colors are taken from Kelly and Judd [Nat. Bur. Stand., Spec. Publ., 440 (1976)] and the accompanying Intersociety Color Council, National Bureau of Standards Centroid Color Charts.

Growth Characteristics

Table III describes the cultural characteristics of culture LL-D42067 on various agar media which were selected from those recommended by the International Streptomyces Project Committee (hereinafter referred to as "ISP").

Micromorphology

Microscopic examination of the strain showed it to form short chains of conidia on aerial hyphae which were slightly hooked to short-spirals (up to three turns). The spore surfaces were smooth when observed by electron microscopy, distinguishing this isolate from *A. verrucosopora*.

Cell Wall Composition

Whole cell analyses showed the strain to contain meso diaminopimelic acid (DAP) and the sugar 3-O-methyl-D-galactose (madurose); thus it falls into whole cell pattern type B. The cell wall composition was of the type III (meso DAP, glutamic acid, alanine, muramic acid and glucosamine) and the phospholipid pattern of type PIV (phosphatidyl ethanolamine and/or methylethanolamine plus unknown glucosamine-containing phospholipids). These data support the assignment of the strain to the genus *Actinomadura*. The PIV phospholipid type is not typical for *A. madurae*, which is usually PI.

Physiological Reactions

The physiological reactions of strain LL-D42067 were examined using both the ISP system, Shirling and Gottlieb [Intern. J. Syst. Bacteriol., 16: 313-340 (1966)] and the Gordon tests, Gordon, et al. [Intern. J. Syst. Bacteriol., 24: 54-63 (1974)]. The utilization pattern of the strain on ISP carbohydrate media is given in Table IV, along with those of other members of the genus reacting similarly. Culture LL-D42067 resembles the *Actinomadura madurae* and *Actinomadura verrucosopora* groups. As indicated above, however, it differs from *Actinomadura verrucosopora* in having smooth spore walls. A comparison of reactions in the Gordon test series of *Actinomadura madurae* (Gordon's data; see reference above) and LL-D42067, summarized in Table V, revealed differences only in amylase production and acid from glycerol and raffinose. Since amylase production and raffinose utilization have been found to be variable in *Actinomadura madurae* [Goodfellow, N., et al., J. Gen. Microbiol., 112: 95-111 (1979)], the glycerol

reaction remains the only physiological difference of LL-D42067 from this taxon.

Since strain LL-D42067 is the same as *Actinomadura madurae* in all properties evaluated except for its glycerol reaction and its PIV phospholipid pattern, it has been assigned to the taxon *Actinomadura madurae* as a subspecies designated *Actinomadura madurae* subspecies *simaoensis*.

TABLE III

Cultural Characteristics of LL-D42067 <i>Actinomadura madurae</i> subspecies <i>simaoensis</i> on ISP Morphological Media			
Agar Medium	Aerial Mycelium	Vegetative Mycelium	Soluble Pigment
Yeast extract, Malt extract (ISP 2)	White, sparse	Medium orange-brown-I53*	None
Inorganic Salts	Colorless	Colorless	None
Starch (ISP 4)			
Glucose Asparagine (ISP 5)	Colorless	Colorless	None
Oatmeal (ISP 3)	Sparse pinkish-white	Light orange-brown-I52*	None

*I = ISCC Color charts

TABLE IV

Comparison of Carbohydrate Utilization Reactions of LL-D42067 With Related <i>Actinomadura</i> spp.			
Carbohydrate	LL-D42067	<i>A. madurae</i> (a)	<i>A. verrucosopora</i> (a) (b)
L-arabinose	+	+	+
D-fructose	+	+	+
D-inositol	-	variable	variable
D-mannitol	+	+	+
Raffinose	-	-	-
rhamnose	+	+	+
sucrose	+	+	+
D-xylose	+	+	+

(a) Goodfellow, M., et al., J. Gen. Microbiol., 112:95-111 (1979).

(b) Nonomura, H. and O'Hara, Y., J. Ferm. Technol., 49:904-912 (1971).

TABLE V

Gordon Test Reactions of LL-D42067		
	LL-D42067	<i>A. madurae</i> (Gordon Data*)
<u>Degradation/Transformation of</u>		
Casein	+	+(98)
Xanthine	-	-
Hypoxanthine	+	+(98)
Tyrosine	+	+(91)
Adenine	-	-
<u>Production of</u>		
Amylase	-	+
Gelatinase	+	+
Phosphatase	-	ND
Nitrate Reductase	+	+(98)
Urease	-	-
Esculinase	+	+(98)
<u>Growth on/in</u>		
5% Sodium Chloride	-	ND
Salicylate	-	ND
Lysozyme Broth	-	-(91)
<u>Utilization</u>		
Acetate	+	+
Benzoate	-	-(94)
Citrate	-	+(83)
Lactate	+	ND
Malate	+	+(84)
Mucate	-	-
Oxalate	-	ND
Propionate	-	ND
Pyruvate	+	ND
Succinate	+	+(83)
Tartrate	-	-
<u>Growth at</u>		
10° C.	-	-

TABLE V-continued

Gordon Test Reactions of LL-D42067		
	LL-D42067	<i>A. madurae</i> (Gordon Data*)
45° C.	+	-(66)
53° C.	-	-
<u>Acid from</u>		
Adonitol	+	+(91)
Arabinose	+	+
Cellobiose	+	+
Dextrin	+	ND
Dulcitol	-	-
Erythritol	-	-
Fructose	+	ND
Galactose	+	+(84)
Glucose	+	+
Glycerol	-	+
Inositol	-	+(60)
Lactose	-	+(55)
Maltose	-	+(53)
Mannitol	+	+
Mannose	+	+(94)
Melibiose	-	-
α -Methyl-D-glucoside	-	-
Raffinose	variable	-
Rhamnose	+	+
Salicin	+	ND
Sorbitol	-	-
Sucrose	+	ND
Trehalose	+	+(96)
Xylose	+	+
β -Methyl-D-xyloside	+	ND

*Percentages of cultures showing reaction given in parentheses if not 100%.
ND = Not determined.

For the production of this new antibacterial and anti-parasitic agent the present invention is not limited to this particular organism or to organisms fully answering the above growth and microscopic characteristics, which are given for illustrative purposes only. In fact, it is desired and intended to include the use of naturally-occurring mutants of this organism as well as induced mutants produced from this organism by various mutagenic means known to those skilled in the art such as exposure to nitrogen mustard, X-ray radiation, ultraviolet radiation, N'-methyl-N'-nitro-N-nitrosoguanidine, actinophages and the like. It is also desired and intended to include inter- and intraspecific genetic recombinants produced by genetic techniques known to those skilled in the art such as, for example, conjugation, transduction and genetic engineering techniques.

The in vitro antimicrobial spectrum of LL-D42067 α was determined by the agar plate dilution method with Mueller-Hinton agar and an inoculum of each test organism of approximately 10⁴ colony forming units delivered by the Steers replicating device. The minimal inhibitory concentration (MIC) in mcg/ml was defined as the lowest concentration of LL-D42067 α that inhibited visible growth after 18 hours incubation at 35° C.

The results, summarized in Table VI, show that LL-D42067 α was active versus gram-positive bacteria and moderately active against yeasts.

TABLE VI

Antimicrobial Spectrum of LL-D42067 α		
Test Organism		MIC (mcg/ml)
<i>Staphylococcus aureus</i>	Smith	\leq 0.06
<i>Staphylococcus aureus</i>	LL #14	\leq 0.06
<i>Staphylococcus aureus</i>	LL #27	\leq 0.06
<i>Staphylococcus aureus</i>	LL #45	\leq 0.06
<i>Staphylococcus aureus</i>	ATCC 25923	\leq 0.06
<i>Staphylococcus epidermidis</i>	CMC-83-56	\leq 0.06
<i>Staphylococcus epidermidis</i>	ATCC 12228	\leq 0.06
<i>Streptococcus faecalis</i>	ATCC 29212	\leq 0.06

TABLE VI-continued

Antimicrobial Spectrum of LL-D42067 α		
Test Organism		MIC (mcg/ml)
5 <i>Streptococcus (enterococcus) sp</i>	OSU-75-1	\leq 0.06
<i>Streptococcus (enterococcus) sp</i>	SM-77-15	\leq 0.06
<i>Streptococcus mutans</i>	ATCC 27352	\leq 0.06
<i>Streptococcus mutans</i>	BHI (b)	\leq 0.06
<i>Streptococcus sanguis</i>	G9B (a)	\leq 0.06
<i>Micrococcus luteus</i>	PC 1001	\leq 0.06
10 <i>Bacillus subtilis</i>	ATCC 6633	\leq 0.06
<i>Bacillus cereus</i>	LL #4	\leq 0.06
<i>Candida albicans</i>	CA 300	256
<i>Saccharomyces cerevisiae</i>	Y 15	32
<i>Escherichia coli</i>	#311	512
<i>Escherichia coli</i>	ATCC 25922	512
15 <i>Klebsiella pneumoniae</i>	AD	512
<i>Proteus morganii</i>	K-79-25	512
<i>Acinetobacter calcoaceticus</i>	Stfd-79-17	512

The antibiotic LL-D42067 α derives utility from its antibacterial and antiparasitic activities. For example, the antibiotic may be used in the suppression of intestinal bacterial flora, as a topical antibacterial agent or antiseptic against gram-positive bacteria and as a general disinfectant for surfaces such as instruments. It may also be useful as an antiprotozoal agent in the treatment of malaria. In addition to its antimicrobial and antiparasitic activity LL-D42067 α is effective as an anticoccidial agent in poultry. This utility is the subject of a copending application for U.S. Letters Patent.

In therapeutic use, the compound of this invention may be administered in the form of conventional pharmaceutical compositions appropriate for the intended use. Such compositions may be formulated so as to be suitable for oral or topical administration. The active ingredient may be combined in admixture with a non-toxic pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration, i.e., oral or topical.

General Fermentation Conditions

Cultivation of *Actinomadura madurae* subspecies *simaensis* NRRL 15734 may be carried out in a wide variety of liquid culture media. Media which are useful for the production of this novel antibiotic LL-D42067 α include an assimilable source of carbon, such as dextrin, sucrose, molasses, glycerol, etc.; an assimilable source of nitrogen such as protein, protein hydrolysate, polypeptides, amino acids, corn steep liquor, etc.; and inorganic anions and cations, such as potassium, sodium, ammonium, calcium, sulfate, carbonate, phosphate, chloride, etc. Trace elements such as boron, molybdenum, copper, etc., are supplied as impurities of other constituents of the media. Aeration in tanks and bottles is supplied by forcing sterile air through or onto the surface of the fermenting medium. Further agitation in tanks is provided by a mechanical impeller. An antifoam agent such as silicone oil may be added as needed.

General Procedure for the Isolation of LL-D42067 α

The LL-D42067 α antibiotic is recovered from the fermentation broth by filtration through diatomaceous earth, extracted into a solvent such as methylene chloride and purified by column chromatography on silica gel, using the system hexane:ethyl acetate (80:20) to remove unwanted fats and then methylene chloride:1% acetic acid in methanol (9:1) to give a crude product.

This crude LL-D42067 α is then purified by high performance liquid chromatography on a reverse phase

column using the system acetonitrile:water:acetic acid (600:400:0.28).

EXAMPLE 1

Inoculum Preparation

A typical medium used to grow the primary inoculum was prepared according to the following formula:

Glucose	1.0%
Dextrin	2.0%
Yeast extract	0.5%
N-Z Amine A ^{®1}	0.5%
Calcium carbonate	0.1%
Water qs	100%

¹A pancreatic digest of casein, registered trademark of Sheffield Chemical, Norwich, New York]

This medium was adjusted to pH 7.2 and then sterilized. A 100 ml portion of this sterile medium, in a 500 ml flask, was inoculated with mycelial scrapings from an agar slant of *Actinmadura madurae* subspecies *simaoensis* NRRL 15734. The medium was then placed on a rotary shaker and agitated vigorously at 210 rpm for 48–72 hours at 28° C. This primary inoculum was then used to inoculate 12 liters of the same sterile medium which was then grown at 28° C. for 48 hours providing secondary inoculum.

EXAMPLE 2

Fermentation

A fermentation medium of the following formulation was prepared:

Sucrose	3.0%
Soy flour	1.5%
Corn steep liquor	0.5%
Calcium carbonate	0.5%
Water qs	100%

The medium was sterilized and inoculated at the rate of 12 liters of secondary inoculum from Example 1 per 300 liters of medium. The fermentation was conducted at 28° C. with a sterile air flow of 200 liters per liter of mash per minute, agitation by an impeller operated at 230 rpm for 135–159 hours at which time the mash was harvested and filtered through diatomaceous earth.

EXAMPLE 3

Isolation of LL-D42067 α

The fermentation filtrate from three fermentations, conducted as described in Example 2, were combined, making a total of 1800 liters at pH 7.5, and extracted with 900 liters of methylene chloride. The organic phase was concentrated in vacuo to give 84.1 g of residue.

A 75.2 g portion of this residue was suspended in 300 ml of hexane:ethyl acetate (80:20) and allowed to seep into a glass column (2 inches \times 20 inches) dry packed with silica gel. The column was eluted with a total of 4 liters of the same solvent mixture in order to remove fats and silicone oil and was then eluted with 4 liters of methylene chloride:1% acetic acid in methanol (9:1) collecting 15 ml fractions. The fractions were analyzed by thin-layer chromatography. Antibiotic LL-D42067 α appeared visually as a yellow spot ($R_f=0.5$) with the same solvent system. Fractions 31–60, which contained most of the antibiotic, were pooled and concentrated in vacuo, giving 11.1 g of a red residue.

A 5.5 g portion of the above residue was fractionated by high performance liquid chromatography [Prep LC System-500, Prep PAK-500/C18 cartridge, acetonitrile:water:acetic acid (600:400:0.28), 100 ml/minute, 5.5 g/30 ml/injection]. Thirty 200 ml fractions were collected. Analytical high performance liquid chromatographic analysis of the fractions showed the major portion of LL-D42067 α was in fraction 5. Fraction 5 was allowed to stand overnight. The resulting yellow crystals were collected by decanting off the mother liquor (which was saved), washing the crystals with the mobile phase and air drying, giving 11 mg of LL-D42067 α as yellow crystals.

The mother liquor was concentrated by slow evaporation. The resulting precipitate was collected by centrifugation giving 377 mg of LL-D42067 α as a yellow amorphous solid.

The analytical HPLC conditions were:

Column: μ Bondapak C18, 3.9 mm \times 30 cm, Waters Associates

Mobile Phase: acetonitrile:water:acetic acid (400:600:0.28)

Detector: UV 254 nm and UV 365 nm, 0.2 AUFS

Flow Rate: 1.0 ml/minute

Retention Volume of LL-D42067 α : 11.5 ml.

We claim:

1. A process for producing antibiotic LL-D42067 α which comprises aerobically fermenting the organism *Actinmadura madurae* subspecies *simaoensis*, having the identifying characteristics of NRRL 15734 or an antibiotic LL-D42067 α -producing mutant thereof, in a sterile liquid medium containing assimilable sources of carbon, nitrogen and inorganic anion and cation salts, under operating conditions that are effective for the production of the antibiotic until a substantial amount of LL-D42067 α is produced in the medium and then recovering the antibiotic therefrom.

2. A process for producing antibiotic LL-D42067 α which comprises aerobically fermenting a sterile liquid medium containing assimilable sources of carbon, nitrogen and inorganic anion and cation salts, which medium has been inoculated with a viable culture of the organism *Actinmadura madurae* subspecies *simaoensis*, having the identifying characteristics of NRRL 15734 or an antibiotic LL-D42067 α -producing mutant thereof, maintaining said fermentation culture with sterile aeration and agitation at a temperature of 24°–32° C., at a pH of 7.0–7.6, for a period of 90–200 hours, harvesting the mash and extracting the antibiotic.

3. A biologically pure culture of the microorganism *Actinmadura madurae* subspecies *simaoensis*, having the identifying characteristics of NRRL 15734, said culture being capable of producing antibiotic LL-D42067 α in recoverable quantities upon fermentation in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen and inorganic anion and cation salts.

4. The biologically pure culture of the microorganism *Actinmadura madurae* subspecies *simaoensis* according to claim 3, wherein said microorganism has spontaneously mutated, such that the microorganism is genetically altered but still retains the ability to synthesize antibiotic LL-D42067 α .

5. The biologically pure culture of the microorganism *Actinmadura madurae* subspecies *simaoensis* according to claim 3, wherein said microorganism has been subjected to mutagenic means such that the microorganism is genetically altered but still retains the ability to synthesize antibiotic LL-D42067 α .

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